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Characterization of Protein Structural Changes in Living Cells Using Time-Lapsed FTIR Imaging

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Abstract

Fourier-transform infrared (FTIR) spectroscopic imaging is a widely used method for studying the chemistry of proteins, lipids, and DNA in biological systems without the need for additional tagging or labeling. This technique can be especially powerful for spatially resolved, temporal studies of dynamic changes such as in vivo protein folding in cell culture models. However, FTIR imaging experiments have typically been limited to dry samples as a result of the significant spectral overlap between water and the protein Amide I band centered at 1650 cm^{-1} . Here, we demonstrate a method to rapidly obtain high quality FTIR spectral images at submicron pixel resolution in vivo over a duration of 18 h and longer through the development and use of a custom-built, demountable, microfluidic-incubator and a FTIR microscope coupled to a focal plane array (FPA) detector and a synchrotron light source. The combined system maximizes ease of use by allowing a user to perform standard cell culture techniques and experimental manipulation outside of the microfluidic-incubator, where assembly can be done just before the start of experimentation. The microfluidic-incubator provides an optimal path length of 6–8 μm and a submillimeter working distance in order to obtain FTIR images with 0.54–0.77 μm pixel resolution. In addition, we demonstrate a novel method for the correction of spectral distortions caused by varying concentrations of water over a subconfluent field of cells. Lastly, we use the microfluidic-incubator and time-lapsed FTIR imaging to determine the misfolding pathway of mutant copper–zinc superoxide dismutase (SOD1), the protein known to be a cause of familial amyotrophic lateral sclerosis (FALS).

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Notes

The authors declare no competing financial interest.

Fourier-transform infrared (FTIR) spectroscopy has long been known as a widely used technique for determining changes in protein secondary structure *in vitro* with a high degree of sensitivity^{1–3} as evidenced by its application to the study of many protein-misfolding diseases, such as Alzheimer’s disease,^{4–7} Parkinson’s disease,^{8–10} amyotrophic lateral sclerosis (ALS),^{11–13} and Huntington’s disease.¹⁴ Similarly, mapping or imaging using FTIR microspectroscopy permits the interpretation of these structural features spatially across whole tissue sections or within a single cell.^{15,16} Importantly, protein folding studies using *in vivo* techniques often complement prior *in vitro* studies to yield data taking into account the crowded environment of the cytosol and presence of molecular chaperones.¹⁷

Unfortunately, because of the shared 1650 cm^{-1} vibrational frequency between the O–H bending motion of water and C=O stretching motion of the amide bonds in proteins, samples are typically prepared fixed and desiccated in order to obtain resolvable Amide I data, making the use of the technique for real-time *in vivo* imaging difficult. While substituting water with D₂O presents an attractive option, the exchange of deuterated with atmospheric water over the course of extended experiments adds quantitative complications that mitigate the benefits. Additionally, the use of deuterated media has been shown to affect cellular processes, reduce culture viability to varying degrees based on concentration and duration of acclimation^{18–21} and, especially pertinent to this study, reduced protein structural flexibility,²² which may alter protein folding and unfolding.

Recent advances have slowly addressed the barriers that prevent the application of FTIR imaging to living cells in cell culture models, such as those available for protein-misfolding diseases. The first major advent was the pairing of synchrotron infrared light with commercial FTIR microscopes, providing the necessary brightness to obtain spectra with high signal-to-noise at diffraction-limited spatial resolution.^{23–25} The second major advancement was the emergence and development of cell culturing within microfluidic devices.²⁶ For example, using a commercially available demountable IR liquid cell, Moss, et. al were able to combine these techniques to obtain one of the first detailed FTIR spectra for subcellular features in living cells through aqueous medium, demonstrating the practical application of the technique.³ Further needed improvements in flow-cell design and fabrication achieved a reduced path length of $<10\text{ }\mu\text{m}$, while maintaining cell viability, thereby increasing the IR transmission to obtain high quality FTIR spectra.^{27–30} Lastly, the most recent advancement was the combination of the focal plane array (FPA) detector with a synchrotron IR source and objectives with high magnification and high numerical aperture. This combination has enabled the rapid acquisition of a large field of cells with submicron

pixel resolution and high signal-to-noise.^{30–33} This is an especially critical component to compensate for cell migration and obtain sequential images over a long duration for time-resolved studies. However, to date, no study has demonstrated synchrotron-based, full-field FTIR imaging with submicron pixel resolution to produce time-lapsed FTIR “movies” in biological systems.

Here we demonstrate for the first time the ability to collect sequential FTIR images of living cells over the course of 12 h with subcellular spatial resolution using a synchrotron source and FPA detector. We designed a custom demountable microfluidic-incubator system with a working distance of <1 mm using off-the-shelf CaF₂ windows and a polymer film spacer. The short working distance enabled the use of high magnification (52× or 74×) Schwarzschild objectives, which have been demonstrated to provide enhanced spatial resolution and chemical contrast.^{34,35} An alternative approach to improving spatial resolution is by using attenuated total reflectance (ATR) objectives, which have also been successfully used for live cell imaging.^{36,37} However, the high brightness of the synchrotron source enables the effective use of non-immersion, high NA objectives, which also improve spatial resolution and contrast. We also demonstrate the use of a novel water-correction method needed for accurately processing dozens of images produced for each experiment, typically encompassing a total of 125 000 spectra per data set. To demonstrate the system’s functionality, we then used the system to study the protein aggregation process of mutant copper–zinc superoxide dismutase in familial amyotrophic lateral sclerosis (ALS).

MATERIALS AND METHODS

Cell Culture

Chinese hamster ovary (CHO-K1) cells (ATCC CCL-61) were cultured in Ham’s F-12K (Kaighn’s) medium with 10% fetal bovine serum (Invitrogen), 1% penicillin-streptomycin (Invitrogen) and incubated at 37 °C with 5% CO₂. At approximately 90% confluence, cells were harvested using trypsin-ethylenediaminetetraacetic acid (EDTA) (Sigma-Aldrich, St. Louis, MO) and seeded directly onto the surface of the bottom drilled CaF₂ windows (1 mm thick) by pipetting 50 000 cells dropwise. Cells were incubated under the prior conditions for an additional 24–48 h prior to start of experimentation. This seeding density produces a sparse to midrange confluency (30–50%) well suited for imaging individual cells.

Following culturing on CaF₂, the flow cell was assembled stepwise over a period of 10 min while it was maintained at 37 °C with a warm stage (Linkam Scientific Instruments Ltd.). Fresh media ultrabuffered with 20 mM HEPES, pH 7.2, was loaded into a 5 mL syringe and kept covered at room temperature for at least 12 h prior to start of the experiment to allow for degassing. Excess gas bubbles adhered to the walls of the syringe were dislodged by forceful tapping and then expelled. The media was flowed at a rate of 2.2 μ L/min, calculated to replace the volume of media in the sample chamber approximately twice per minute. Cells were monitored under visible time-lapse microscopy for 20 min post assembly to observe for proper liquid flow in the sample space, consistency in cell morphology, and positive incidence of mitosis.

SOD1 Transfection

CHO cells were plated onto CaF₂ as previously described and, after 24–48 h, were transiently transfected with Lipo-D 293 transfection reagent (SigmaGen) to overexpress mutant D125H SOD1-YFP protein. Cells were incubated for an additional 9 h post transfection under standard conditions prior to assembly of the flow cell. Fluorescent cells were identified and selected via light microscopy using a 10× objective. For this study, individual highly fluorescent cells were chosen for high aggregation potential and ease of distinguishing cytosolic and nuclear compartments when generating regions of interest (ROIs) for data processing.

Stage Incubator and Flow Cell

To keep the cells viable during data collection, a microfluidic-incubator was required with design properties that are not commercially available. In particular, it was necessary to take into consideration the shallow 1.9 and 1.0 mm working distance of the high magnification 52× or 74× Schwarzschild objectives, respectively. The ability to disassemble the flow-chamber was needed to allow for a majority of cell culture work to be performed using standard methods prior to data collection, and allow for modularity in the design to accommodate different window thicknesses. This resulted in the creation of a low-profile, demountable sandwich cell (Figure 1) consisting of two 25 mm diameter CaF₂ windows (Crystran Ltd., U.K.) separated by a 4 μm ultralene spacer (SPEX SamplePrep, LLC) cut to size. The bottom window was 1 mm thick with two drilled 1 mm diameter inlet and outlet ports. The top window was 0.5 mm thick. The sandwiched windows were inserted into a bottom aluminum base plate mated to a modified 2 in. threaded lens tube (ThorLabs SM2L05). A top plate covered the windows and was secured in place with a threaded locking collar. The windows were separated from the body of the flow cell with two 25 μm Teflon gaskets. Liquid feeding rate was set to 2.2 μL/min and controlled by a Harvard 33 twin syringe pump set to push/pull pump to facilitate flow. The temperature was maintained at 37 °C using a warm stage as previously described.

FTIRI Data Collection

After assembly of the liquid flow cell, the apparatus was transported to the beamline for data collection. All FTIR imaging data were collected at beamline U10B at the National Synchrotron Light Source (NSLS), Brookhaven National Laboratory (BNL). Beamline instrumentation consisted of a Bruker Vertex 80 V infrared spectrometer coupled to a Hyperion 3000 infrared microscope equipped with a focal plane array (FPA) imaging detector. A multibeam synchrotron source coupled to the instrument provided high brightness illumination necessary for achieving the high signal-to-noise required for rapid data acquisition through an aqueous medium.³³ We used a high magnification 52× (0.65 NA) Schwarzschild objective (Edmund Optics Inc.) to achieve an effective pixel resolution of 0.77 μm and a field of view of up to 64 × 64 pixels. For the purpose of these experiments, the illuminated field of view was set to 48 × 44 pixels.

Each infrared image was collected in transmission mode between 900 and 3900 cm⁻¹ with a spectral resolution of 8 cm⁻¹ and 256 scans per image. Background subtraction was performed on a cell-free region containing only cell culture medium. A macro written in

Bruker's OPUS 6.5 software automated a collection routine cycling between four image locations—three different cells and a background point. Each individual IR image took approximately 2.75 min to complete and a full macro cycle took 11 min. Visual images were collected simultaneously while epifluorescence images were collected every 3–4 h to reduce potential phototoxicity.

Water Correction

Because of the large water signal, a water correction algorithm was necessary. In particular, since the background spectra were collected from a region that contained cell culture media but no cells, an iterative algorithm was developed in MATLAB (The Mathworks, Inc.) to compensate for the oversubtraction of water in areas containing cells. The algorithm was designed to perform an iterative background correction at every pixel to account for differences in cell thickness and cell migration over time.

The goal of the algorithm is to achieve baseline congruence in the region bordering either side of the alkyl C–H stretch peaks at a loci of 2900 cm^{-1} as quantitatively measured by their slopes, termed S1 and S2 (Figure 2). This baseline becomes significantly distorted upon oversubtraction of water; therefore, an unbiased correction was performed by the readdition of a water spectrum until the difference of slopes S1 and S2 are minimized. The correction was validated through a check of the Amide I/II ratio as measured by the absorption intensity at 1650 and 1550 cm^{-1} , respectively. The appropriate Amide I/II ratio was determined from fixed CHO cells and found to fall between 1.35–1.6.

The water subtraction algorithm was first tested using a simulated water-oversubtracted aqueous FTIR map of CHO cells (Figure 3). In this test, a FTIR image from a field of dried, fixed CHO cells was used to create the simulated map. At each pixel, a standard water spectrum was subtracted by a random factor between 0.08 and 0.30 of the normalized maximum intensity at 1650 cm^{-1} . Then the algorithm was applied in order to correct for the oversubtracted water at each pixel, ideally with the addition of water at the same factor used for the subtraction. Quantification of the error was determined based on the method of Dousseau et al.³⁸ Specifically, the control (dried, fixed cells), corrected, and uncorrected spectra were first normalized to a total integrated intensity value of 1.0 between 1480 and 1720 cm^{-1} . Then the percent error was determined from the difference in intensity values at each frequency. This was calculated for each pixel and averaged ($n=2500$ pixels). The average error in the Amide I band region from $1700\text{--}1600\text{ cm}^{-1}$ was found to be 2.2% (range = 0.4–2.4%). The algorithm was then applied to a FTIR image of the living cells, producing an Amide I integration map with spectral features consistent with that of control cells.

RESULTS AND DISCUSSION

The narrow path length of the flow cell has the potential to cause significant difficulty maintaining cell health during long measurement periods, which can last more than 24 h post assembly. Therefore, we assayed the CHO cells for viability using trypan blue to expose necrotic and dead cells in 6 h increments for 24 h after flow cell assembly (each time point is a separate experiment and performed with 3 repetitions). A total of 200 cells were

counted and the percentage of viable cells was assessed based on color change (Figure 4). The counterpart control experiments were performed on cells cultured on CaF₂. Start time was initiated upon replacement of media with 20 mM HEPES ultrabuffered medium and cultured in an incubator devoid of supplemental CO₂.

Control CHO cells cultured under standard incubation conditions showed a very small loss in viability ($0.6\% \pm 0.4$). In contrast, the cells maintained in the flow-cell showed a slightly higher reduction in viability ($3.1\% \pm 1.2$) after 24 h of incubation. This small decrease in viability can be attributed to phototoxic effects from exposure to visible light, shearing forces from the flow of medium, and possible reduced oxygenation of medium through degassing.²⁶ These results are consistent with other published reports using microfluidic devices to maintain cell cultures, typically showing little cell death and lack of cell-wall permeability after several hours.^{27,28,39}

We demonstrated a practical application of the technique by examining the process of protein aggregation in a cell culture model of amyotrophic lateral sclerosis (ALS). ALS is a neurodegenerative disorder affecting the motor neurons in the spinal cord, brain stem, and motor cortex of patients with the disease, causing loss of muscle control throughout the body. Although most cases of ALS are sporadic in nature, the disease can be inherited through mutations in the protein copper–zinc superoxide (SOD1), an antioxidant protein responsible for the elimination of superoxide (O₂^{•−}) from cells in the body. While mutant SOD1 aggregates are known to be toxic to the motor neurons of patients with the disease,⁴⁰ the mechanism behind the aggregation processes and cause of cytotoxicity is not clearly understood. Prior in vitro studies have produced conflicting evidence for proposed SOD1 aggregation pathways. One explanation proposes a direct aggregation pathway from nascent, unfolded protein,⁴¹ while others show evidence for an unfolding process from fully folded or partially folded SOD1.⁴² Since actual cellular environments may affect protein folding and aggregation differently than in vitro models,¹⁷ obtaining in vivo evidence can greatly clarify the true pathway.

SOD1 is a β -barrel homodimer composed of eight antiparallel β -sheets, comprising 37% of the protein structure.⁴³ Changes in tertiary structure during unfolding and aggregation events are reflected in IR vibrational modes at 1695 cm^{−1} for antiparallel β -sheets, at 1645 cm^{−1} for unordered structure, and at 1630 cm^{−1} for a combination band from both parallel and antiparallel β -sheets. Cerf et al. have shown that by calculating the 1695/1630 cm^{−1} ratio, corresponding to the proportion of antiparallel to parallel β -sheet structure, it is possible to distinguish between the soluble oligomeric and insoluble fibrillar forms of misfolded amyloid β -peptide (A β) linked to Alzheimer's disease.⁴⁴ In addition, the determination of high antiparallel β -sheet structure in oligomeric A β led to a new model of tertiary and quaternary A β organization and possible explanation for the associated cytotoxic effects observed in the disease.⁴⁵

For these experiments, we used a cell culture model of ALS to transiently transfect CHO cells to overexpress mutant D125H SOD1 with a yellow fluorescent protein (YFP) fusion tag for identification and selection of positively transfected cells. The D125H mutation has a slow propensity for aggregation over a 24 h period after initial transfection and shows

reduced structural stability derived from a poor ability to bind zinc and copper to its metal centers.⁴⁶ Cells producing high intensity fluorescence were selected and imaged for 12 h post assembly.

Data produced were water corrected and processed to produce time-lapse “movies” for changes in β -sheet structure (Figure 5). By calculating the intensity ratio at 1695/1630 cm^{-1} and plotting as a false-color map with an overlaid visible image, the protein aggregates were found to have a high antiparallel β -sheet structure within the cytosol (Figure 5A). Averaged spectra and second derivatives from protein-rich regions in the cytosol at 11.5 and 24.5 h also demonstrated an increase in β -sheet structure (Figure 5B). Plotting this ratio over time from 12 to 24 h post-transfection, a decrease in antiparallel β -sheet contribution was observed (Figure 5C). By ratioing the intensities at 1695, 1645, and 1630 cm^{-1} to the integrated area of Amide I, changes to the antiparallel β -sheet, unordered, and parallel/antiparallel β -sheet combination bands as individual components were generated (Figure 5D). Linear correlations were drawn by the method of least-squares to within 95% confidence intervals.

We observed an increase in the combination band over time while the antiparallel β -sheet band showed little change, indicating an increase in total parallel β -sheet structure during the aggregation process. A similar change in the profile of amide I has been shown previously by Valentine et al. for induced amyloid-like SOD1 aggregation in vitro, which demonstrated a sharp decrease in the antiparallel β -sheet band and a peak shift in the combination band.¹¹ In the same study, the pathway from soluble SOD1 to the formation of amyloid-like aggregates was shown by a shift in the circular dichroism (CD) spectra characteristic of amyloid formation. These spectra demonstrated increases in β -sheet and irregular secondary structure. This type of change in β -sheet structure is often associated with fibril formation as found for amyloid beta in Alzheimer’s disease.⁴⁷

Further, unordered structure (1645 cm^{-1}) also showed an increase over the same time frame. This indicates an increase in unfolded protein over the course of the experiment. Computational studies of localized unfolding and aggregation affinity by Bille et al. showed unmetallated wild-type SOD1 displays a strong preference for the formation of parallel intermolecular β -sheet interactions.⁴⁸ This is in contrast to results published for the G37R mutant that showed an increase in antiparallel β -sheet structure over time, suggesting the formation of pore-forming oligomeric structure during aggregation.⁴⁹ The differences in the spectroscopic signatures for these two mutation types bring evidence that multiple pathways can exist for the aggregation of SOD1 in vivo.

CONCLUSIONS

The production of time-lapse FTIR images at subcellular spatial resolution is a powerful tool for studying localized time-dependent intracellular changes in protein structure in vivo. For the first time, we demonstrated the development and application of extended-duration, subcellular spatial resolution FTIR imaging of living cells in aqueous medium using a FPA detector, a 52 $\times$ objective, a synchrotron infrared source, and a novel technique for water correction. The method for water correction shown here enables accurate and rapid

automated processing of the large data sets inevitably generated by these experiments. This new methodology was used to understand the misfolding pathway of SOD1-ALS in cell culture and can fill gaps in knowledge for many other neurological protein misfolding diseases, including Parkinson's disease (alpha-synuclein), Alzheimer's disease (amyloid-beta), Huntington's disease (huntingtin), and the prion protein diseases, such as Creutzfeldt-Jakob disease, chronic wasting disease, and mad cow disease.

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Figure 1.

Flow cell assembly. (A) A 0.5 mm thick CaF_2 top window and 1 mm thick bottom window are separated by a spacer patterned from 0.4 μm Ultralene film, and sandwiched between two aluminum plates. Three pegs are used to align the plates and prevent torsion while securing the retaining ring. (B) The bottom window has two 1 mm drilled holes to pass medium through the sample space. (C) Assembled flow cell.

Figure 2.

Spectral features used for the water correction. Specifically, a water spectrum is added at each pixel to minimize the difference between slopes S1 and S2 (inset). The correction was validated using the amide I/II ratio, which was 1.35–1.6 as determined empirically from fixed, dried cells.

Figure 3.

Water correction over an entire FTIR image. (A) Visible image of the CHO cells. Amide I integration maps (B) before and (C) after water correction (common color scale). (D) The correction factors plotted spatially show good alignment to the sample map, with the greatest correction applied to the densest cellular regions. (E) Spectra from a selected pixel (inset box) before and after water correction. Scale bars in panels A–D are 10 μm .

Figure 4.

Cell culture viability assay. (A) CHO cells maintained in the flow cell exhibit slightly lower viability relative to control cells. (B) Viability was assessed using 0.4% trypan blue solution added to adhered cells and counted after 10 min of exposure. Dead and necrotic cells swell and stain blue after addition of the solution. Scale bar in B is 50 μm .

Figure 5.

(A) D125H aggregation process at 11.5 and 24.5 h (top and bottom rows, respectively). The visible, fluorescence, and antiparallel/parallel β -sheet ratio (1695/1630 cm^{-1}) map are shown left to right. Scale bar is 10 μm . (B) Averaged spectra and second derivatives from protein-rich regions in the cytosol at 11.5 and 24.5 h. (C) Ratio of 1695/1630 cm^{-1} bands plotted as percent difference as a function of time (slope = -0.37 ± 0.17). (D) Ratios of the 1695, 1645, and 1630 cm^{-1} bands to the Amide I area were generated and percent differences from the original time point were plotted as a function of time (slopes = -1.23 ± 0.22 , 0.37 ± 0.13 , and 0.27 ± 0.11 , respectively). Linear regressions were performed by least-squares analysis and uncertainty is depicted by 95% confidence intervals. From panels C and D, results showed a decrease in antiparallel β -sheet content, an increase in the combination band, and an increase in unordered structural motifs suggesting the formation of protein fibrils connected by parallel β -sheet linkages for the D125H mutant.